FORMULATION AND EVALUATION OF SELF-EMULSIFYING DRUG DELIVERY SYSTEMS OF AZITHROMYCIN TO ENHANCE ITS PENETRATION THROUGH THE EPITHELIAL TIGHT JUNCTIONS

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by

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
°C	Celsius
µg	Microgram
µl	Microliter
µm	Micro-meter
ANOVA	Analysis of variance
AZM- $F1_{(H)}$	Optimized azithromycin incorporated Liquid self-emulsifying drug delivery system
AZM- FS_{-A200}	Optimized azithromycin incorporated Solid self-emulsifying drug delivery system
AZM-L-SEDDS	Azithromycin incorporated liquid self-emulsifying drug delivery system
AZM-S-SEDDS	Azithromycin incorporated solid self-emulsifying drug delivery system
Blank $F1_{(H)}$	Optimized blank liquid self-emulsifying drug delivery system
Blank FS_{-A200}	Optimized blank solid self-emulsifying drug delivery system
Caco-2	Continuous line of heterogeneous human epithelial colorectal adenocarcinoma cells
cm	Centimeter
cm^2	Squared centimeter

CO_2	Carbon dioxide
D.H_2O	Distilled water
DC	Drug content
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
DS	Droplet size
FBS	Foetal bovine serum
GIT	Gastro Intestinal Tract
h	Hour
HCl	Hydrochloric acid
HLB	Hydrophilic lipophilic balance
HPLC	High performance liquid chromatography
I.M	Intramuscular
I.V	Intravenous
LOD	Limit of detection
LOQ	Limit of quantification
L-SEDDs	Liquid self-emulsifying drug delivery system
m^2	Square metre
mg	Milligram
mg/ml	Milligram per millilitre
min	Minute
ml	Milliliter
ml/min	Milliliter per minute
mM	Millimole

MTT	Microtiter tetrazolium assay
mV	Millivolt
NaOH	Sodium hydroxide
nm	Nano-meter
PdI	Polydispersity index
Pen-Strep	Penicillin-Streptomycin solution
PTFE	Polytetrafluoroethylene
RH	Relative Humidity
rpm	Revolutions per minute
RSD	Relative Standard Deviation
S.C.	Subcutaneous
SD	Standard deviation
SEDDs	Self-emulsifying drug delivery system
SEM	Standard error of the mean
SIF	Simulated intestinal fluid
Smix	Surfactant/co-surfactant ratio
S-SEDDs	Solid self-emulsifying drug delivery system
T %	Transmittance percent
TEER	Trans-epithelial electrical resistance
TEM	Transmission electron microscope
UK	United Kingdom
USA	United States of America
USFDA	United States Food and Drug Administration
USP	United States pharmacopoeia
UV	Ultraviolet

v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
ZP	Zeta potential
Ω	Ohm

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FORMULASI DAN PENILAIAN SISTEM PENGHANTARAN DRUG AZITROMISIN PENGEMULSIAN-KENDIRI UNTUK MENINGKATKAN PENEMBUSANNYA MELALUI PERSIMPANGAN KETAT EPITELIUM

ABSTRAK

Azithromisin (AZM) adalah antibiotik makrolida yang digunakan untuk rawatan beberapa jangkitan bakteria. Ia diketahui mempunyai bioavailabiliti oral rendah (37%) disebabkan oleh berat molekulnya yang tinggi, kelarutan tidak lengkap dan /atau kebolehtelapan usus yang rendah, yang berpotensi menyekat penyerapan usus. Untuk mengatasi kekurangan ini, sistem penghantaran drug cecair pengemulsiaan-kendiri dan pepejal (L-SEDDs dan S-SEDDs) AZM telah disediakan dalam usaha untuk meningkatkan keterlarutan, dan mengubah penembusan selular yang mungkin meningkatkan bioavailabiliti oral. Lapan gambarajah pseudo-ternari yang berbeza dibina berdasarkan kelarutan AZM dan kajian emulsifikasi dalam pelbagai jenis SEDD eksipien pada nisbah surfaktandan ko-surfaktan (Smix) yang berbeza. Kriteria pemilihan ialah ukuran titisan (DS) <150 nm, polidispersiti (PdI) ≤ 0.7, dan transmitan (T)% > 85 dalam tiga pencair, air suling (D.H_2O), 0.1 mM HCl dan simulasi usus cecair (SIF), serta kandungan drug tertinggi. Formulasi akhir L-SEDD (F1 $_{(H)}$), yang terdiri daripada Capryol 90®, Tween 20®, dan Transcutol HP® masing-masing sebanyak 22.22%, 51.85% dan 25.93% (v/v) boleh memenuhi cirri-ciri pemilihan dan mempunyai DS 141.57 ± 1.1 nm, PdI 0.52 ± 0.004, T% 90.1 ± 0.1, dan kandungan drug 60.42 ± 0.4 mg/ml ($p < 0.05$). Oleh itu, ia telah dipilih untuk penukaran kepada S-SEDDs menggunakan agen pemejalan yang berlainan. Aerosil 200® menghasilkan S-SEDD (FS-$_{A200}$) dengan DS paling kecil 155.3 ± 1.91 nm, PdI 0.62 ± 0.03, dan kandungan drug 38.79 ± 0.52 mg/g (p nilai-nilai < 0.05). Formula

blank $F1_{(H)}$ dan FS-$_{A200}$ terbukti selamat pada kepekatan yang berbeza dalam ujian sitotoksisiti microtiter tetrazolium (MTT). Blank dan AZM yang digabungkan dalam formulasi $F1_{(H)}$ dan FS-$_{A200}$ mampu memodulasi ekalapis sel Caco-2 dengan mengurangkan rintangan transepithelial berbanding AZM tulen. Tambahan pula, formulasi AZM-$F1_{(H)}$ dan AZM-FS-$_{A200}$ telah meningkatkan pembebasan AZM dalam D.H_2O, 0.1 mM HCl, dan SIF jika dibandingkan dengan AZM tulen dengan pelepasan> 90% dalam 5 minit dan 60 minit masing-masing oleh AZM-$F1_{(H)}$ dan AZM-FS-$_{A200}$ ($p < 0.05$). Kajian kestabilan menunjukkan bahawa formulasi AZM-$F1_{(H)}$ dan AZM-FS-$_{A200}$ stabil dalam keadaan penyimpanan peti sejuk dengan jangka hayat anggaran masing-masing sebanyak 39.29 dan 40.29 bulan. Kesimpulannya, kedua pengemulsian-kendiri sistem penghantaran drug bermuatan azitromicin dalam bentuk cacair dan pepejal telah berjaya menigkatkan kelarutan dan kadar pelarutan apabila dibandinkan dengan drug bebas tanpa formulasi. Formulasi ini telah terbukti selamat dan berupaya membuka persimpangan ketat transepitelial yang akan meningkatkan pengankutan paraselular.

FORMULATION AND EVALUATION OF SELF-EMULSIFYING DRUG DELIVERY SYSTEM OF AZITHROMYCIN TO ENHANCE ITS PENETRATION THROUGH THE EPITHELIAL TIGHT JUNCTIONS

ABSTRACT

Azithromycin (AZM) is a macrolide antibiotic used for the treatment of a number of bacterial infections. It is known to have a low oral bioavailability (37 %) due to its relatively high molecular weight, incomplete solubility and/or poor intestinal permeability, which is potentially restricting its intestinal absorption. To overcome these drawbacks liquid and solid self-emulsifying drug delivery systems (L-SEDDs and S-SEDDs) of AZM were prepared in an attempt to enhance its solubility, and altering its cellular penetration, which might improve its oral bioavailability. Eight different pseudo-ternary diagrams were constructed based on AZM solubility and emulsification studies in different SEDDs excipients at different surfactant to co-surfactant (Smix) ratios. The selection criteria was droplet size (DS) < 150 nm, polydispersity index (PdI) ≤ 0.7, and transmittance (T) % > 85 in three diluents of distilled water ($D.H_2O$), 0.1 mM HCl, and simulated intestinal fluids (SIF) as well as highest drug content. A final L-SEDDs formulation ($F1_{(H)}$), which is composed of Capryol 90®, Tween 20®, and Transcutol HP® at the concentrations of 22.22 %, 51.85 %, and 25.93 % (v/v) respectively, was able to meet the selection criteria and had a DS of 141.57 ± 1.1 nm, PdI 0.52 ± 0.004, T% 90.1 ± 0.1, and drug content of 60.42 ± 0.4 mg/ml ($p < 0.05$). Accordingly, it was selected for conversion to S-SEDDs using different solidifying agents. Aerosil 200® produced S-SEDDs ($FS\text{-}_{A200}$) with the smallest DS of 155.3 ± 1.91 nm, PdI 0.62 ± 0.03, and drug content of 38.79 ± 0.52 mg/g ($p < 0.05$). Blanks of $F1_{(H)}$ and $FS\text{-}_{A200}$ formulations proved to be safe at different

concentrations upon evaluating them in the microtiter tetrazolium (MTT) cytotoxicity assay. Blank and AZM incorporated $F1_{(H)}$ and FS-$_{A200}$ formulations were able to cross through the Caco-2 cell monolayer through reducing their transepithelial electrical resistance in comparison to the pure AZM. Furthermore, AZM-$F1_{(H)}$ and AZM-FS-$_{A200}$ formulations have increased the release of AZM in D.H_2O, 0.1 mM HCl, and SIF when compared to the pure AZM with a fast release of > 90% in 5 min and 60 min by AZM-$F1_{(H)}$ and AZM-FS-$_{A200}$ respectively (p values < 0.05). The stability study revealed that AZM-$F1_{(H)}$ and AZM-FS-$_{A200}$ formulations are stable at refrigerator storage conditions with estimated shelf life of 39.29 and 40.29 months respectively.

In conclusions, both azithromycin loaded liquid and solid self-emulsifying drug delivery systems have successfully enhanced azithromycin's solubility, and dissolution rate in comparison to the free azithromycin. The formulations were proven safe and capable of opening the transepithelial tight junctions, which would enhance the drug paracellular transport.

CHAPTER 1

INTRODUCTION

1.1 Routes of drug administration: oral drug delivery

A wide range of pharmaceutical and biotechnological products as well as natural compounds are delivered through various routes of administrations such as oral, parenteral (I.V., S.C. and I.M. injections) and topical. The parenteral route is mainly limited by the patient's discomfort, sometimes being expensive, and complications due to un-sterile tools; which leads to infections at the side of administration (Jin et al., 2015). On the other hand, oral route's products represent two-third of the pharmaceutical dosage forms, as it is a more convenient and comfortable route of drug administration, economical and often safer, and requires no special training to take the medication (Al-Achi et al., 2013).

In some cases, achieving therapeutic levels of drugs by oral route may represent a complicated process, which includes overcoming some challenges like drug poor solubility, low bioavailability, instability in gastric acid medium, drug-food interaction, and poor absorption through the gastrointestinal tract (GIT) mucosal barrier (Ensign et al., 2012; Lambkin & Pinilla, 2002). The proper design of an oral dosage form can successfully overcome most if not all of that.

1.2 Biopharmaceutical classification system (BCS)

The U.S. Food and Drug Administration (USFDA) classified oral drugs in bio-pharmaceutical classification system (BCS) into four classes based on its aqueous solubility, and intestinal permeability (as shown in figure 1.1). Practically, a biopharmaceutical behaviour of a drug is determined by studying its aqueous solubility at different pH values ranging from 1 to 6.8. Its dissolution rate is identified by using one of the U.S. Pharmacopeia (USP) apparatus that are specifically selected along with certain dissolution conditions to suit the tested drug based on its USP dissolution profile. The intestinal permeability can be observed on different experimental levels either *in vitro* (human or animal tissue, as well as suitable epithelial cell monolayer cultures), *in situ* intestinal perfusions, or *in vivo* (humans or animal models) (USFDA, 2015).

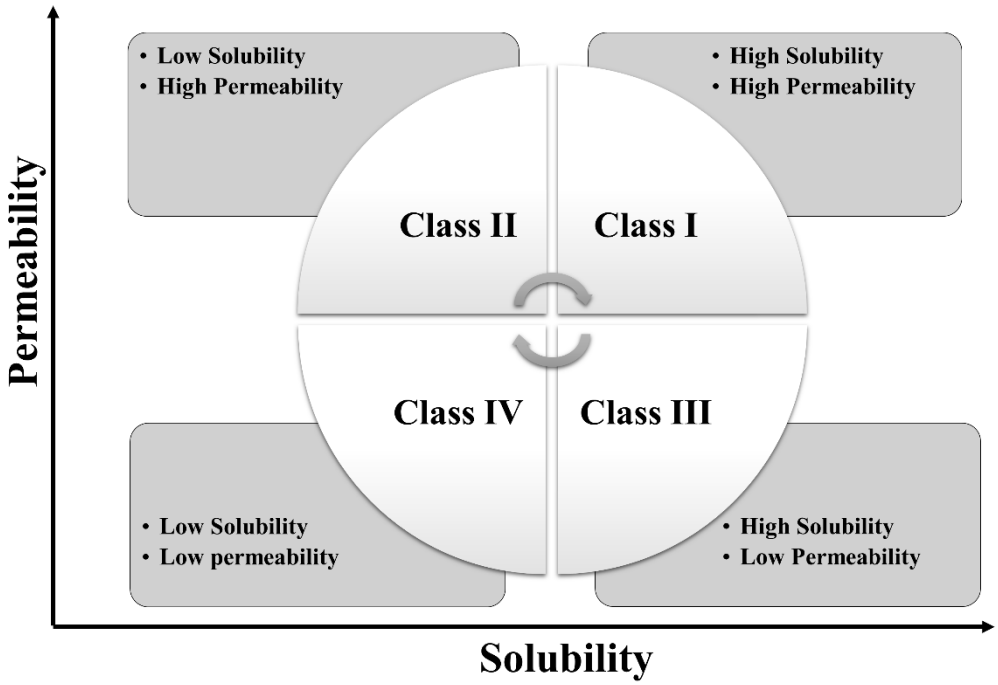

Figure 1.1: The biopharmaceutical classification system of drugs (BCS)

According to the BCS classification, class I drug substances does not require bioavailability and bioequivalence studies due to their high dissolution rate and bioavailability. However, drug in class II and class III require bioavailability and bioequivalence studies. This is because the drugs under class II have a high permeability but low solubility, and low dissolution rate, which in turn lead to a poor oral bioavailability. While the drugs in class III, have low and variable bioavailability due to poor penetration in the intestinal membrane, although the dosage forms have good solubility and dissolution rate. Class IV is suggested to be not suitable for oral dosage form, due to its low solubility and permeability (Ku, 2008; Junghanns & Müller, 2008).

1.3 Classification of the lipid based drug delivery systems

Creativity in applying natural and synthetic oils, lipids and fatty acids to improve solubility of poorly soluble drug and bioavailability has generated remarkable experimental outcomes that lead to academic and commercial successes. One of the earliest definitions of lipid-based formulations is an oil/surfactant mixture (Pouton, 1985). Various literatures were linking the beneficial impacts of fatty food or oils on drug substances bioavailability (Piscitelli et al., 2011; Sim et al., 2005; Humberstone & Charman, 1997; Gourlay et al., 1989). The first classification of this lipid based formulations was introduced in 2000 (Pouton, 2000), while in 2006 additional class was added (Pouton, 2006).

1.3.1 Formulation of type I systems

The drug substance in this formulation is dissolved in a vegetable oil or a medium chain triglyceride (MCT), diglycerides and/or mono-glycerides based on the drug

degree of hydrophobicity. Usually, type I materials is labelled as "generally regarded as safe" (GRAS). The triglyceride is rapidly digested by pancreatic enzyme forms a colloidal dispersion having a coarse particle size. This will enhance intestinal transcellular absorption, and hence drug's bioavailability. For many years, oil-soluble vitamins (A and D) were administered through this system. However, this formulation is restricted for highly lipophilic drugs (Pouton, 2000; Pouton, 2006).

1.3.2 Formulation of type II system

This formulation involves type I constituents, in addition to a lipophilic surfactant with hydrophilic-lipophilic balance (HLB <12). Excipients with medium chain, mono, di and/or tri-glycerides, shows superior results in terms of stability if surfactant concentration is more than 25 %w/w in the formula. However, increasing the surfactant concentration to more than 65 %w/w turns the final formulation into a viscous liquid crystalline phase with slower emulsification time. Following the oral intake, the digestion of this type will form a turbid oil/water dispersion (o/w), with a particle size ranging from 0.25 to 2 µm (Pouton, 2006). Nevertheless, the type II system has not attracted much attention within the pharmaceutical industry. This might be due to the commonly used surfactants for type II formulation, which are not included in the USFDA list of inactive ingredients (Sarita et al., 2012).

1.3.3 Formulation of type III systems

In this formulation, both hydrophilic and hydrophobic components were used (Pouton & Porter, 2008). Usually, surfactants with HLB >12 are employed either surfactant with the primary type I lipid materials, or surfactant, co-solvents and the primary type

I lipid materials. Some literatures split type III into two sub-categories including type IIIA, which contains less water-soluble surfactants and/or co-solvents, and type IIIB, which contains a higher portion of water-soluble contents (Holban & Grumezescu, 2016; Pouton, 2006). The particle size range of this type of formulations is 100 to 250 nm, with clear or almost clear dispersion. However, if the surfactant content is increased ≥ 40 % (under gentile agitation conditions), fine dispersions of <100 nm might be produced (Constantinides, 1995). Drug substances with lipophilicity partition coefficient (log P) from 2 to 4 showed an increase in the formulation solvent capacity and optical clarity. Type III formulations were named as self-emulsifying drug delivery system (SEDDs). SEDDs have been used successfully to overcome the biopharmaceutical drawbacks of many drugs. Consequently, various drugs are available in the market in SEDDs form such as Sandimmune® and Neoral® (Cyclosporin A), Fortovase® (Saquinavir) and Norvir® (Ritonavir) (Robert, 2007).

1.3.4 Formulation of type IV systems

These formulations have totally excluded the presence of the oil phase, and entirely depend on the selection of a proper mixture between hydrophilic surfactants and co-solvents producing formulations with a particle size range from 50 to 100 nm. Upon dilution, a micellar solution is formed instead of dispersion due to the dominated presence of surfactant, which can lead to solvent capacity loss (Holban & Grumezescu, 2016; Pouton & Porter, 2008). The co-solvent role is to facilitate the surfactant's dispersion and to reduce the irritancy that might be caused by the high surfactant concentrations in some formulations. These systems are compatible with few drugs, a commercial example of this type is the capsule of the HIV protease inhibitor

amprenavir (Agenerase®, GSK) (Strickley, 2004). The typical composition of various types of lipid formulations is listed in table 1.1

Table 1.1: The typical composition of various types of lipid formulations (Pouton, 2006)

Excipients in formulation	**Content of lipid based formulation (%, w/w)**				
	Type I	**Type II**	**Type IIIA**	**Type IIIB**	**Type IV**
Oils: triglycerides or mixed mono and diglycerides	100	40 - 80	40 - 80	<20	-
Water-insoluble surfactants (HLB < 12)	-	20 - 60	-	-	0 -20
Water-soluble surfactants (HLB > 12)	-	-	20 - 40	20 - 50	30 -80
Hydrophilic co-solvents	-	-	0 - 40	20 -50	0 - 50

1.4 Self-emulsifying drug delivery system (SEDDs)

Out of the four discussed types of lipid-based formulations, self-emulsifying drug delivery system (SEDDs) are the most used colloidal dispersions in the experimental and commercial fields (Kalepu et al., 2013; Hauss, 2007). Prior to formulation, the proper selection of SEDDs constituents is highly essential in order to know each component's properties, and employ them to match the selected drug nature and the intended research aim. Drug solubility in the nominated excipients is the first parameter to consider, along with pseudo ternary diagram construction.

1.4.1 Advantages and disadvantages of SEDDs

SEDDs were found to have many advantages, including increasing drug solubility, enhancing its dissolution rate (Gurram et al., 2015), and increasing membrane fluidity to facilitate transcellular absorption either through reducing the trans-epithelial electrical resistance (TEER) through either opening the tight junction (Sha et al., 2005), or inhibiting cytochrome P450 (CYP450) enzymes (Zhao et al., 2013; Trivedi et al., 2013). The anhydrous nature of the formula will add further advantages to it, including long-term stability, patient compliance, ease of manufacturing and scale up. The limitation of SEDDs is summarized in their high content of surfactants and co-surfactants (Smix), which increased the safety concerns. Thus, conducting cytotoxicity studies for almost every designed formula is recommended. The high surfactant/co-surfactant mixture (Smix) ratio might lead to drug's degradation and instability (Shobhit et al., 2012).

1.4.2 Dilution impact on SEDDs behaviour

Upon dilutions, SEDDs spontaneously form a nano or micro emulsion based on the formulation design, HLB values, materials and drug's physiochemical nature. The formed nano-emulsions are having a stable particle size upon different dilution ratios, ranging from10 to 300 nm, and considered kinetically stable. Its formation is highly dependent on the components mixing order (a surfactant first mixed with an oil) at room temperature. On the other hand, micro-emulsions are highly dependent on the thermodynamic variables such as temperature and composition. A wide range of particle structures can be detected within a one micro-emulsion involving the formation of many phases at equilibrium (up to three) in the same flack, that have different types of nano-metric scaled morphologies (Kahlweit et al., 1990) including

worm-like, bi-continuous sponge-like, liquid crystalline, or hexagonal, spherical swollen micelles, Anton & Vandamme, (2011).

1.4.3 SEDDs constituents and their role

The SEDDs constituents' selection are based on general parameters such as purity, stability, cytotoxicity and irritancy, solvents capacity and miscibility. Other properties such as self-dispersion, digestibility and the safe elimination of the digested products are also needed to be considered during material selection. Moreover, using low cost materials will always be an additional point, Pouton & Porter, (2008).

1.4.3(a) Oil

Naturally, oil phase plays a critical role in solubilizing a lipophilic drug or isolated substance, and facilitating self-emulsification. Besides that, oil also boosts the GIT absorption of the drug by increasing its transport via the intestinal lymphatic system (Gershanik & Benita, 2000; Lindmark et al., 1995; Charman & Stella, 1991), thus, altering the drug biopharmaceutical properties. Researchers have used long, medium and short chain fatty acids with different degrees of saturation (Caliph et al., 2000). The edible type of vegetable oils showed a low capacity to dissolve drugs, while the modified or hydrogenated vegetable oil were more commonly used, as they have higher capability to dissolve drugs, and formed good emulsification systems (Gupta et al., 2013; Constantinides, 1995). Moreover, semi-synthetic medium chain derivatives with amphiphilic nature and surfactant properties, are progressively replacing the regular medium chain triglyceride oils in the SEDDs, Neslihan & Benita, (2004).

1.4.3(b) Surfactants

Various types of surfactants with different HLB values ranging from 2 to 18 have been used for SEDDs development, Rajan & Nirav, (2011). Their usage boosts drug dissolution rate and cellular permeation Neslihan & Benita (2004). Safety is the major concern to be considered in surfactants selection. Natural emulsifiers are safer than synthetic ones, but they have limited emulsification capacity (Constantinides, 1995). Non-ionic surfactants (with high HLB values) are dominating other surfactants types within SEDDs formulation because they are less toxic compared to ionic surfactants (Seema et al., 2016; Swenson et al., 1994). Usually, stable SEDDs are containing a surfactant concentration between 30 to 60%. Increasing surfactant concentration will create a dispersion with a smaller particle size, but after a certain limit, particle size will start to increase (Kommuru et al., 2001).

1.4.3(c) Co-surfactants and co-solvents

The addition of a co-surfactant to the SEDDs formulation may help to reduce the amount of surfactant used especially if a high surfactant concentration is needed. The synergistic surfactant/co-surfactant effect would lower the interfacial tension to form fine droplets (Swain et al., 2016).

Co-solvents are used to induce SEEDs homogeneity, stability, and to increase the solubility of the incorporated drug in the designed formula, they usually enhance the hydrophilic surfactants dispersion in the oil phase. Medium length chain alcohols (C8 – C12) are usually selected for SEEDs studies, along with ethylene-glycol, glycerol, and propylene glycol derivatives (Swain et al., 2016).

1.4.4 Mechanism of self -emulsification

The full description of the self-emulsification process is still not clear. A self-emulsified dosage forms is a mixture of an oil and surfactant, whereby the addition of water it will form an emulsion with little or no energy input. Such emulsion will deliver the drug to the GIT in an emulsified form that creates a high surface area for dissolution, hence improves drug bioavailability (Craig et al., 1995).

During the formation of a classical emulsion, the excess surface free energy is dependent on the droplet size and the interfacial tension. Surfactants are employed to stabilize the emulsion and prevent the phase separation via reducing the interfacial tension and the free energy (Craig et al., 1995). However, in SEEDs formation, the free energy is very low, hence resulted in thermodynamic spontaneous emulsification. Scientists suggested that self-emulsification occurs due to water penetration into the liquid crystalline (LC) phase that is formed at the oil/surfactant-water interface and during gentle agitation. This LC phase is probably the reason behind the high stability of the resulting nano-emulsion against coalescence (Groves & de Galindez, 1976; Wakerly et al., 1986).

1.4.5 Conversion of liquid SEDDs to solid SEDDs

Liquid SEDDs (L-SEDDs) could be converted into solid state SEDDs (S-SEDDs) which can be formulated in different pharmaceutical dosage forms such as tablets, pellet or capsules. This conversion is made to combine the advantages of both L-SEDDs and S-SEDDs in one dosage form. S-SEDDs are known for their low production cost, convenience of process control, high stability, reproducibility and patient compliance. Researchers have adopted various techniques to obtain S-SEDDs

including spry drying, adsorption to solid carriers (Tang et al., 2008), extrusion spheronization (Fanun, 2016), and melt granulation (Chambin et al., 2004).

1.4.6 Food impact on the absorption of drugs loaded SEDDs

SEDDs lipidic constituents are similar to dietary lipids existing in daily food. Usually, larger quantities of lipid (> 2 g) are capable of stimulating additional bile secretion; hence increase the luminal concentration of bile. This will provide a lipidic microenvironment for forming emulsion droplets, that will be transformed into various components such as vesicular and micellar phases (Kollipara & Gandhi, 2014). Researchers reported that the administration of poorly soluble drugs in SEDDs formulations would make the drug less sensitive toward food intake compared to the drugs in their pure forms (Woo et al., 2008; Nielsen et al., 2008). Cinnarizine SEDDs capsules and SEDDs tablets were capable of reducing the food effect on the drug absorption in dogs (Christiansen et al., 2014). The concomitant administration of blank SEDDs with cinnarizine tablets to 10 healthy volunteers was able to reduce the food effect on the absorption of the drug (Christiansen et al., 2016).

1.5 Azithromycin

Azithromycin (AZM) is a semi-synthetic 15-membered macrolide antibiotic and the first azalide in the market with a superior antibacterial activity among its family members for the last three decades. The drug was approved by USFDA in 1991. AZM was synthesized in an attempt to produce an acidic stable macrolide, with a wider bacterial spectrum, and longer half-life (Imperi et al., 2014).

1.5.1 Physiochemical properties

AZM (chemical structure in figure 1.2) is an amorphous powder, slightly water soluble (2.37 mg/L at 25 ˚C), with a melting point of 114°C, and LogP of 4.02 as well as a pKa of 8.74 (at 25 ˚C) (U.S. National Center for Biotechnology Information, 2016). The anhydrous AZM is having a relatively large molecular weight of 749.0 Da (Luke & Foulds, 1997).

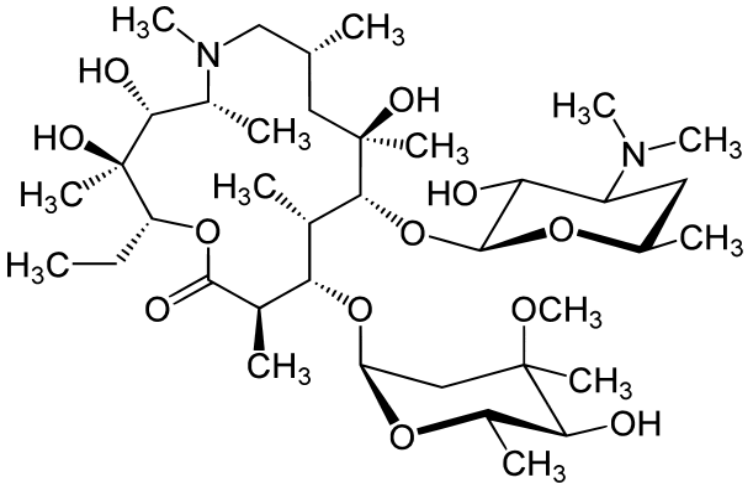

Figure 1.2: Structure of azithromycin

1.5.2 Biopharmaceutical classification (BCS) of AZM

The World Health Organization (2005) described AZM biopharmaceutical classification as either Class III (high aqueous solubility with low permeability), or Class IV (poor aqueous solubility with poor permeability). Moreover, Gandhi et al. suggested that AZM should be in class III of the biopharmaceutical classification. The authors made their conclusion based on the results obtained by reviewing the published literatures about AZM in the Medline (Gandhi et al., 2014).

1.5.3 Pharmacological actions

AZM is active against a wide range of gram-positive and gram-negative organisms (Peters et al., 1992; Williams, 1991). It is the drug of choice for the treatment of community-acquired pneumonia (Kuzman et al., 1995), typhoid fever (Butler et al., 1999), *Shigella dysenteriae* (Niyogi, 2005), trachoma (Chen et al., 2010), non-gonococcal urethritis (Carlin & Barton, 1996), and skin infections (Mallory, 1991). AZM inhibits the protein synthesis in bacteria by binding to the 50s ribosomal subunit and preventing the translocation of peptides (Bekaert & White, 2006), which accordingly inhibits the RNA dependent protein synthesis (Learning, 2012).

The oral intake of AZM is associated with `GIT adverse effects, including diarrhoea/loose stools, nausea, and abdominal pain. About 1-2% of the patients under AZM medication experienced elevations in ALT (SGPT), AST (SGOT), or gamma-glutamyltransferase (GGT, GGTP). Intravenous infusion of AZM may cause adverse effects include pain at the injection site or local inflammation occurred in 6.5 % or 3.1 % of patients respectively (U.S. National Center for Biotechnology Information, 2016).

1.5.4 Pharmacokinetics

AZM has a wide therapeutic index and low bioavailability of 37 %. The oral dosing regimen is 500 mg daily for three days, or alternatively 500 mg as initial dose followed by 250 mg daily for a period of four days (Naieni & Akrami, 2006). AZM is metabolized hepatically by demethylation, (Schlagenhauf-Lawlor, 2007). AZM elimination is via bile excretion (Kee et al., 2014), it is largely found unchanged in urine and stool (Yaffe & Aranda, 2010). AZM tissue concentrations exceed serum

concentrations by 10- to 100 folds, with a slow release pattern of AZM from tissues, leading to a very long half-life (68 h), and an elimination half-life of 3 days. Such profiles permit once a day dosing with a short treatment period in many cases (Katzung et al., 2012; Pfizer Laboratories, 2016).

The drug absorption capacity within the small intestine is linked with the accessibility of it to the GI mucosal surface, where absorption occurs Arcangelo & Peterson (2006). Food intake was found to have different impacts on AZM based on its consumed pharmaceutical dosage form. Administration of AZM capsules with food reduced its rate of maximum concentration (C_{max}) by 52 %, and the extent of the area under the curve (AUC) by 43 %. In contrast, food intake with AZM oral suspension, increased the C_{max} and the AUC by 46 % and 14 % respectively. While the intake of AZM tablets with food increased the drug tolerability, and the C_{max} by 31%, but the AUC remained unchanged (Pfizer Laboratories, 2016; USFDA, 2012).

1.6 Literature review

1.6.1 Nanotechnology based techniques in enhancing AZM oral solubility and bioavailability

Various nanocarriers were investigated to enhance AZM oral delivery and improve its anti-bacterial properties. AZM conjugated with neutral polyamidoamine dendrimers were more influential than the conventional AZM against *Chlamydia trachomatis* at its persistent infectious form (Mishra et al., 2011). AZM microcapsules with an average diameter of 1.2 µm had higher dissolution rate compared to the pure AZM (Zhang et al., 2010). While AZM microspheres with a larger particle size (11.65 – 14.45 µm) were also able to improve the drug dissolution profile (Li et al., 2012).

Other studies observed that AZM nano suspension prepared using different polymers had higher dissolution rate and a sustained release property (Kaushik et al., 2015) with a particle size of 200 nm and improved drug solubility (Hou et al., 2012). Zhang et al found that AZM-nanosuspension size of 400 nm showed a better release performance than the micro scale one (Zhang et al., 2007).

Nanoparticles of AZM with a particle size of 212 to 252 nm prepared using different ratios of poly(lactide-co-glycolide) (PLGA) polymer were found to improve AZM potency against a wide range of bacteria by modifying AZM surface characteristics, its adsorption to the bacterial cells and uptake (Mohammadi et al., 2010; Azhdarzadeh et al., 2012). Noble metals nanoparticles of AZM silver (15 to 30 nm), and gold (20 to 40 nm) were proven safe, and were able to increase AZM antibacterial activity against *E.coli* and *Staphylococcus aureus* (Namasivayam & Samrat, 2016; Namasivayam & Ganesh, 2012).

Darabi et al. reported that some AZM nanotubes were able to enhance its penetration in a *Micrococcus luteus* cell culture model, and increase the antimicrobial activity (Darabi et al., 2014). The bioavailability of AZM was also enhanced by using a cationic niosomes-based delivery systems (0.95 – 5.87 µm) (Zhong et al., 2014). Recently, AZM-nanofibers (100-300 nm) and nanobeads (100-500 nm) were developed using Eudragit® RS100 polymer, and were able to boost AZM minimum inhibitory concentrations, and increased its antimicrobial activity against *S. pneumonia* (Adibkia et al., 2016).

1.6.2 L-SEDDs and S-SEDDs in drug delivery

L-SEDDs (liquid SEDDs) and S-SEDDs (solid SEDDs) are utilized and studied to produce advanced dosage forms of conventional drugs; and they ended up as marketed products under the licence of leading companies within the pharmaceutical industry. L-SEDDs and S-SEDDs have enhanced drug's performance through improving their solubility, bioavailability and permeability. These nanocarriers have reduced the administered doses of different drugs by improving their C_{max}, which in turn reduced the side effects of loaded drugs in comparison to their conventional forms. The rapid onset of action of L-SEDDs and S-SEDDs offer faster drug therapy. This is particularly important in cases where the fast pharmacological action is needed such as in inflammation, hypertension and angina conditions (Nepal et al., 2010). S-SEDDs formulations are preferred from pharmaceutical point view over L-SEDDs formulations as they provide more flexibility to formulate a wider range of dosage forms. Nevertheless, until now there is no clear conclusion whether L-SEDDs or S-SEDDs is superior for oral drug delivery based on the conflicting results that were reported in the literatures. In an *in vivo* study, L-SEDDs showed superiority results in the delivery of cyclosporine A in comparison to its S-SEDDs form (Kim et al., 2001). Another study showed that both the S-SEDDs and L-SEDDs of progesterone had similar *in vitro* and *in vivo* profiles (Tuleu et al., 2004). The *in vitro* assay revealed that the efficacy of L-SEDDs and S-SEDDs was similar for cilnidipine (Bakhle & Avari, 2015), and fenofibrate (Shazly & Mohsin, 2015). Fexofenadine loaded L-SEDDs and S-SEDDs had similar potentials in increasing the oral bioavailability through enhancing the dissolution rate, blocking the P-glycoprotein efflux pump, and CYP450 hepatic metabolism (Trivedi et al., 2013). The L-SEDDs were superior to S-SEDDs in term of physiochemical properties when loaded with atorvastatin. However, in this

case S-SEDDs showed a much better dissolution rate indicating better solubilisation properties (Kosnik et al., 2015).

In general, L-SEDDs and S-SEDDs are considered as stable lipidic formulations (Wang et al., 2008). S-SEDDs were stable for the period of three months at room temperature as well as at 40 °C (Jaiswal et al., 2014). L-SEDDs and S-SEDDs loaded with tacrolimus were evaluated for enhancing bioavailability and their shelf life in two different studies at 25 °C. Shelf life in the first study was 1.84 and 2.25 years (Patel et al., 2013); while in other study it was 1.76 and 2.27 years, respectively (Hitesh et al., 2012). Moreover, both types of formulations had similar efficacy in enhancing drug's bioavailability. Thus, it can be concluded that the properties of L-SEDDs or S-SEDDs are very much influenced by the constituent's nature, HLB value, properties of drug to be incorporated and preparation method.

1.6.3 Marketed SEDDs formulations

The first marketed drug in the form of SEDDs was cyclosporine A (Sandimmune®), and later other products were also introduced into the market, including ritonavir (Norvir®), as well as Saquinavir (Fortovase®). These formulations had shown to improve the bioavailability of drugs significantly compared to the conventional formulations (Hu & Li, 2011). Examples of the marketed pharmaceutical products formulated as self- emulsifying systems (SEDDs) are shown in table 1.2.

Table 1.2: Examples of pharmaceutical products formulated as self-emulsifying drug delivery systems Rajan & Nirav (2011)

Drug name	Compound	Dosage form	Company
Neoral	Cyclosprorine	Soft gelatin capsule	Novartis
Norvir	Ritonavir	Soft gelatin capsule	Abbott laboratories
Fortovase	Saquinavir	Soft gelatin capsule	Hoffmann-La Roche lnc.
Agenerase	Amprenavir	Soft gelatin capsule	Glaxosmithkline
Solufen	Ibuprofen	Hard gelatin capsule	Sanofi- Aventis
Lipirex	Fenofibrate	Hard gelatin capsule	Sanofi- Aventis

1.7 Problem statement

The effort to improve the solubility and dissolution of a poorly water-soluble drug remains one of the most challenging tasks in drug development. Azithromycin low solubility in water and biological fluids is considered as the major contributing factor to its erratic and low dissolution rate as well as its low bioavailability (37 %) after oral administration; this is mostly linked to its hydrophobic nature and high molecular weight. Such low oral bioavailability leads to a high oral dose of azithromycin, longer terms of medication and greater related gastrointestinal side effects. On the cellular level, azithromycin was reported to have a tendency to increase the tight junctions closing through increasing the transepithelial electrical resistance values, hence affected the azithromycin paracellular transport, and reduced its intestinal permeability.

Furthermore, I.V injection, the only alternative route of drug administration, has been associated many side effects, including erythema, pain, swelling and tenderness at the site of injection. SEDDs formulations had shown promising results in improving wide range of lipophilic drug delivery through enhancing their solubility, dissolution rate, bioavailability, and altering their cellular penetration mechanisms. Thus, SEDDs could have a potential in improving the oral delivery of azithromycin.

1.8 Objectives

The objectives of this study are to:

I. Develop and validate a stability indicating HPLC method for azithromycin (AZM) in bulk and AZM loaded self-emulsifying drug delivery system (AZM-SEDDs).

II. Design and characterize the AZM-SEDDs in the form of liquid and solid formulations.

III. Investigate the ability of liquid and solid AZM-SEDDs to enhance AZM solubility, and dissolution rate in different PH values.

IV. Evaluate the developed liquid and solid AZM-SEDDs formulations safety on a Caco-2 model.

V. Investigate the ability of the AZM-SEDDs to reduce the transepithelial electrical resistance tight junctions on a Caco-2 model.

VI. Evaluate the stability and shelf life of the liquid and solid formulations of AZM-SEDDs

CHAPTER 2

DEVELOPMENT AND VALIDATION OF A STABILITY INDICATING HPLC METHOD FOR THE QUANTIFICATION OF AZITHROMYCIN IN SELF-EMULSIFYING DRUG DELEIVERY SYSTEM

2.1 Introduction

The ultraviolet-visible (UV-Vis) spectrophotometry is the most widely used quantitative method in the pharmaceutical analysis due to its simplicity and rapidness (Siladitya et al., 2012). However, azithromycin (AZM) ultraviolet (UV) detection is highly challenging due to the absence of the conjugated double bond in its lactone ring, which consequently results in a low molar absorptivity (Kanfer et al., 1998). Thus, it is necessary to develop an HPLC method for the quantification of AZM in pure and self-emulsifying drug delivery system (AZM-SEDDs) forms for the *in vitro* release, and further studies.

Different techniques have been used to quantify AZM in various biological fluids and pharmaceutical dosage forms including LC–MS/MS (Shen et al., 2010), thin layer chromatography-densitometry method (Kwiecień & Gadek 2013; Khedr & Sheha, 2003), cyclic voltammetry and square-wave voltammetry methods using different electrodes (Peng et al., 2011; Avramov et al., 2006; Farghaly & Mohamed, 2004; Nigović & Šimunić 2003) and Raman spectroscopy quantification by measuring drug's peak intensity (Shende et al., 2014). However, these methods are either expensive, tedious or time consuming in comparison to a simple HPLC-UV method.

Based on literature review, some HPLC-UV methods have been developed to detect AZM in pharmaceutical dosage forms (Shaikh et al., 2008; Kulikov & Verushkin, 2004). However, only few methods were reported under stability indicating conditions (Subbareddy, 2015; Ramesh M., 2012; El-Gindy et al., 2011; Al-Rimawi & Kharoaf, 2010). Moreover, despite the fact that HPLC-UV techniques were used to quantify AZM in some nano-formulations, but the validation of these methods was not mentioned (Zhong et al., 2014; Pouretedal, 2014; Yan et al., 2009).

Accordingly, the aim of this work is to develop a simple, specific and reproducible stability indicating HPLC–UV method for the determination of AZM in bulk, AZM-SEDD and in different in *vitro release* mediums. The method was developed and validated as per the recommendations of the International Conference on Harmonization ICH guideline (ICH, 2005).

2.2 Materials and methods

2.2.1 Materials

Azithromycin anhydrous (purity > 97.2%) was a kind gift from Wockhardt Research Centre (Aurangabad, India). Acetonitrile and methanol (HPLC grade) were purchased from J.T. Baker (Phillipsburg, USA). Ammonium acetate was bought from Bendosen Laboratory Chemicals (Bendosen, Norway). Tween 20® (Polyethylene glycol sorbitan monolaurate) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), Capryol 90® (Propylene glycol monocaprylate) and Transcutol HP® (Diethylene glycol monoethyl ether) were purchased from Gattefossé (Lyon, France). All other chemicals used were of analytical reagent grade and obtained commercially.

2.2.2 Instrumentation

The study was conducted on a Shimadzu liquid chromatography system (VP series, Kyoto, Japan) with CBM/20A system controller, LC/20AD solvent delivery pump, SPD/20A UV/VIS detector, SIL/20A auto-sampler, and CTO/10ASvp oven system. Data acquisition and analysis were performed using Shimadzu LabSolutions® software (version 5.30 SP1) (Kyoto, Japan).

2.2.3 Chromatographic condition

The chromatographic separation was performed using a Hypersil GOLD C-18 analytical column with the dimensions of 250 mm x 4.6 mm ID x 5 µm, Fisher Scientific (Waltham, Massachusetts, USA). The flow rate was set at 0.7 ml/min, and the detection wavelength was set to 210 nm. The oven temperature was maintained at 60 ºC, and an injection volume of 10 µl was employed. Mobile phase was consisting of acetonitrile: ammonium acetate solution (30 mM, pH = 6.8) (82:18 v/v). The mobile phase was filtered through nylon membrane filter 0.45 µm Titan®, Thermos Scientific (Waltham, Massachusetts, USA), sonicated and degassed before used. All samples were filtered before injection using syringe filter 0.2 µm Pall (New York, USA).

2.2.4 Preparation of stock solution, calibration standards and quality control samples

The standard stock solution of AZM (5000 µg/ml) was prepared by dissolving 50 mg of AZM powder in 10 ml of the methanol and sonicated in ultrasonic bath (Branson 5510, USA) for 2 min. Then it was further diluted with the diluting solution (which consist of acetonitrile: ammonium acetate solution (60:40 v/v) to obtain a working standard solution of 1600 µg/ml. Solutions for the calibration were prepared by

diluting the working standard solution with the diluting solution to give concentrations in the range of 5 to 200 µg/ml. Three quality control (QC) solutions at low (LQC), medium (MQC) and high (HQC) concentrations were prepared of 15, 100 and 180 µg/ml, respectively.

2.2.5 System suitability studies

System suitability tests were conducted to verify the performance and the reproducibility of the chromatographic system. Analytical parameters such as retention time (R_t), theoretical plates (N), tailing factor (T), and resolution (R_s) were checked at the three QC concentrations in six injection replicates.

2.2.6 Specificity

The specificity test is a reflection of the analytical method capacity in measuring the drug in the presence of impurities, excipients, and degradation products (if any). The test was done by comparing the chromatograms of the mobile phase, AZM standard solution, blank-SEDD, AZM-SEDDs, *in vitro* release mediums, 0.1 mM HCl solution (pH = 4), and simulated intestinal fluid (SIF, pH = 6.8).

2.2.7 Stress degradation studies

The stress degradation studies were done to measure the ability of the developed method to assess unequivocally the analyte in the presence of components such as degradation products, impurities, and excipients (ICH, 2005). In this study, the stress degradation conditions of acid, base, oxidation, heat and light were performed on four solutions namely: AZM standard, AZM-SEDD, blank-SEDD and mobile phase. The AZM standard solution of 1600 µg/ml was prepared by diluting the required volume

of the standard stock solution with methanol. A volume of 0.27 ml of AZM-SEDD (60 mg/ml AZM) was dissolved in methanol to get the AZM concentration of 1600 µg/ml. The blank-SEDD solution was prepared similar to AZM-SEDD solution. The mobile phase was used as it is.

2.2.7(a) Acid and alkali degradation studies

For acid degradation study, two sets of four 10 ml flasks were filled in with 1 ml of AZM standard, AZM-SEDD, blank-SEDD or mobile phase, respectively. Subsequently, 1 ml of 1 mM HCl was added to all the flasks. The solutions in the first set were neutralized immediately with 1 ml of 1 mM NaOH, then the diluting solution was added up to 10 ml. These solutions were served as zero hour samples. The solutions in the second set were left on the bench at room temperature (25 ± 2 ºC/65 ± 5 % relative humidity) for 24 h, then neutralized and diluted by the same way. These solutions were served as 24 h samples. All the samples were injected in triplicate.

For alkali degradation study, similar procedure was used, but 1 ml of 100 mM NaOH was added to each flask instead of 1 ml of 1 mM HCl and the neutralization procedures were done using 100 mM HCl.

2.2.7(b) Oxidative hydrogen peroxide (H_2O_2) degradation

Two sets of four 10 ml flasks were filled in with 1 ml of AZM standard, AZM-SEDD, blank-SEDD or mobile phase, respectively. Subsequently, 1 ml of 3 % H_2O_2 was added to all the flasks. The solutions in the first set were immediately diluted with diluting solution up to 10 ml. These solutions were served as zero hour samples. The solutions in the second set were left on the bench at room temperature (25 ± 2 ºC/65 ± 5 %